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Review

The self-organization of lipids and proteins of myelin at the membrane interface. Molecular factors underlying the microheterogeneity of domain segregation

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ABSTRACT

The advances over the last 10 years on the understanding of myelin heterogeneity are reviewed. The main focus is on the applicability of Langmuir monolayers, Langmuir–Blodgett films and some associated techniques to unravelling the behaviour of interfaces formed with all the components of a natural membrane. Lipid–protein lateral segregation appears as a major driving force to determine surface patterns that can change under compression from circular domains to two-dimensional fractal structures. The major proteins of the myelin membrane induce lateral segregation in an otherwise homogeneous surface formed by the mixture of total myelin lipids. The lipid and protein components appear to distribute in the surface domains according to their charge, compressibility and relative molecular weight: myelin proteins, ganglioside GM1 and fluorescent lipid probes partition into liquid-expanded phase domains; other components such as phosphatidylserine and galactocerebroside partition into another liquid phase enriched in cholesterol. Simplified protein–lipid mixtures allow assessment of the participation of the major proteins in the two dimensional pattern development. One of the major myelin proteins, the Folch–Lees proteolipid, self-segregates into, and determines formation of, fractal-like patterns. The presence of the second major protein, myelin basic protein, leads to round liquid-expanded domains in the absence of Folch–Lees proteolipid and softens the boundaries of the fractal structures in its presence. The location of myelin basic protein in the interface is surface pressure sensitive, being slightly squeezed out at high surface pressure, allowing the fractal domains enriched in Folch–Lees proteolipid to evolve.

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1. Monolayers at the air/water interface as biomembrane models

The idea of forming physically controlled monomolecular films on an aqueous surface initiated with the work of Benjamin Franklin as accounted to the proceedings of the Royal Society in 1774 [1] and continued to emerge over the end of the 19th and beginning of 20th century (see ref. [2] for a historical perspective). Organized layers of amphipathic molecules (beginning with fatty acids) were described by using X-ray diffraction of crystals [3] and by analyzing surface phenomena related to the existence of molecular layers at the air/liquid interface [4–6]. Such films at the air/aqueous interface have now been used for at least 80 years as an experimental approach to model biomembranes [7]. In fact, this important early work (which actually led to the very idea of the lipid bilayer as the structural unit of biomembranes), was performed with complex lipid extracts of the whole erythrocyte membrane from several mammals. Thus, the history of natural membrane monolayers is an old one, but at the same time is quite short because most of the interest over subsequent decades was focused only on more simple and chemically well defined monolayers. These simplified systems contributed seminal ideas to the biomembrane field, many of which are still appealing and under active investigation, such as the presence of coexisting segregated surface domains determining lateral heterogeneity [8–10], phase transitions [11–13], the condensing effect of cholesterol [14], the interfacial penetration/adsorption of proteins [15,16], liquid–liquid immiscibility [17], the critical unilamelar state of biomembranes [18], among many others.

Attempts to form compositionally complex monolayers with whole natural membranes (including their proteins), while deriving some meaningful (supra-)molecular information, were absent for several years until they slowly reappeared over the last three decades [19–23]. This pioneering work opened the possibility of working, (even if in a semi-quantitative manner) with monolayers having the whole chemical complexity of a natural membrane, including all or most of its lipids and proteins, and the inherent advantages of the monolayer as a well controlled biointerface in terms of molecular packing and lateral surface pressure (see below). However, until recently, this type of studies was not aimed at analyzing the surface behaviour in terms of domain arrays (including shape, size, distribution and composition), or their superstructuring [10], in correlation with defined molecular properties or interactions among the components forming the monolayer. Here we review recent studies on this topic and, as a general base, will mostly focus on the features of myelin monolayers at the air/buffer interface.

2. Why monolayers?

This question has been addressed a number of times, see for instance ref. [16]. Briefly, the advantage of performing studies on monolayers lies in the fact that the thermodynamic state can be precisely defined and manipulated. Parameters such as temperature, surface pressure, molecular area, surface potential, compressibility, number of coexisting phases, dipole moment can be directly and simultaneously measured in classical monolayer studies; the thermodynamics of mixing is also quite straightforward to analyze [8]. Besides, some of these parameters are much more difficult to obtain (or they are essentially inaccessible) in the more popular and putatively more biologically appealing bilayers.

On the other hand, due to new instrumental developments, experimentation with the monolayer system broadened its scope during the last 25 years. Of particular importance was the pioneering use of fluorescence microscopy techniques to directly observe monolayers at the air/water interface. Monolayers can be doped with fluorescent lipid probes that partition preferentially among different phases [24], providing contrast to study the lateral heterogeneity. Moreover, dedicated new optical techniques were developed, like Brewster angle microscopy (BAM), a non-invasive technique which gains contrast from differences in reflectivity among the different phases [25,26]. Information about the relative optical thickness of the film can be obtained from local reflectivity measurements [27,28], while absolute values can be obtained by ellipsometry [29]. Additionally, monolayers can be transferred from the air/water interface to solid supports by the classical Langmuir–Blodgett and Langmuir–Schaefer techniques [30] and the different lipid and protein components can be labelled and localized by immunolabelling [31]. This brief enumeration represents just a few of the varied techniques that can be actually applied to monolayer studies. There are increasing possibilities from sophisticated emerging techniques becoming increasingly available (see for instance the book edited by Möbius and Miller [32], and ref. [33]). Application of a number of such techniques to films with a heterogeneous, but defined, lipid–protein composition offers interesting opportunities to study complex membrane systems under controlled and known surface organization. This has direct implications for an understanding of the lateral organization, morphology and superstructuring of phase segregated domains.

3. Why myelin?

For a decade we have studied monolayers formed from whole bovine spinal cord (central nervous system) myelin, as well as some of its fractions and reconstituted mixtures. From a practical point of view, myelin offers some advantageous features to approach the exploration of the surface properties of a complex lipid–protein interface containing most of the components of a natural membrane. On one hand, it is relatively easy to obtain in large quantities with high purity. It is also the membrane with the lowest protein content (25% w/w) relative to lipids, with about 80% of them represented by two major protein kinds, the Folch–Lees proteolipid (PLP) [34] and the myelin basic protein (MBP) [35] fractions. Both proteins are soluble in organic solvents [36] and, perhaps for this reason, the whole membrane becomes soluble in them. To our knowledge, this lipid–protein solubility is shared only by lung surfactant [37,38]. Regarding cell membranes, such solubility is unique for myelin and, in fact, it constitutes a purity criterion [39]. This represents a clear advantage for quantitative monolayer spreading at the air/water interface because the surface pressure–molecular area compression isotherms can be directly calculated from the spread amount [8,40]. More generally, monolayers can also be spread from aqueous biomembrane suspensions [19,22,23,40,41] by applying the method of Trurnit [42], commonly used for the spreading of hydrophilic polymers and proteins [43]. Therefore, the solubility in organic solvents is not a necessary condition for achieving quantitative biomembrane spreading. However, the spreading from aqueous suspensions requires considerable more work in order to obtain the molecular parameters because the tiny amounts of material remaining at the interface must be collected in sufficient quantity and precisely quantified for that purpose; this approach is rather laborious, time consuming and the possible errors can be large [23,40,41]. Finally, the adsorption of interfacial (Gibbs) films from myelin vesicles in the subphase showed the natural tendency of myelin to adsorb to the air/water interface [44], and offered the possibility of working with mono- or multi-layered interfaces [40,45]. In summary, in order to explore the surface properties of a complex lipid–protein interface containing most of the components of a natural membrane, myelin shows some advantageous physico-chemical properties over other membranes. Nevertheless, by properly adjusting the experimental conditions, this type of approach can in principle be amenable to use with other biomembranes [19,22,41].

Compact myelin[1] (the bulk myelin with typical periodicity of about 160 Å), once it is assembled by glia cells, shows considerable structural stability. Alteration of its state by moderate physical perturbations (osmotic stress, for instance) is generally reversible. All this suggests that myelin is a membrane whose structure is rather close to its thermodynamic equilibrium state, and this makes the system simpler to interpret. It is worth mentioning that from about 50 tissues dissected from the Tyrolean Ice Man (dead 5000 years ago) myelin was the only tissue well preserved, chemically as well in its fine structure at the electron microscope level, with periodicities comparable to those of normal myelin [46].

Finally, myelin is a long standing "biophysicist's" membrane. This is not just because of the above mentioned simplicity but also because of its structural periodicity. In this work, we will not review studies on conductivity, nerve impulse propagation, membrane order perturbation (anaesthetics) and other functional biophysical aspects but we will concentrate on its supramolecular structure. Classically, these types of studies have been mostly focused on the structure of the membrane along its transverse section; this has been a natural choice, because the myelin multilayer spiral is periodic in that direction which makes it amenable to diffraction studies. Thus, the transverse structure of myelin is rather well understood and has served as a model system for the development of diffraction techniques to deduce information about the bilayer structure of biomembranes in general [47] leading for instance to the idea of bilayer asymmetry. These kinds of studies were successfully correlated with cryo-fracture electron microscopy experiments [48] which rendered detailed information about its supramolecular architecture, even in nearly *in vivo* conditions in nerve.

In this work we do not concentrate on the transverse structure of myelin but on the lateral organization adopted by the myelin lipids and proteins when spread as a monolayer at the air–aqueous interface. It is worth emphasizing that, although this system is a valid model for a compositionally complex interface that can be studied under controlled and known molecular packing, caution should be exercised when attempting to extrapolate the conclusions reached from monolayers to *in vivo* bilayer membranes. There are several drawbacks, for instance the asymmetry of the membrane is lost, the interactions between myelin surfaces are also lost and the hydrophobic thickness is reduced, which creates a different environment to which the conformation of transmembrane proteins must adapt. Nevertheless, it should be noted that similar problems can arise when working with reconstituted bilayer systems; for instance, having PLP incorporated into the "proper" myelin lipid bilayer environment does not guarantee having the native structure of PLP [49,50].

4. The existence of domains

Having shown the possibility of spreading and of studying some thermodynamic properties of monolayers having identical composition to that of whole myelin on one hand [40], and the advent of microscopy techniques on the other, the following simple question can be directly addressed: are these films homogeneous? From the single collapse surface pressure observed and the absence of cooperative surface pressure-induced liquid-expanded to liquid-condensed transition in the myelin monolayer [40] the more likely scenario was to expect a homogeneous surface. Nevertheless, the first microscopic observation of a freshly prepared monolayer from a natural cell plasma membrane (Fig. 1), containing all the components of whole myelin, indicated a rich and complex heterogeneity along the lateral plane [51]. The study employed the standard method using

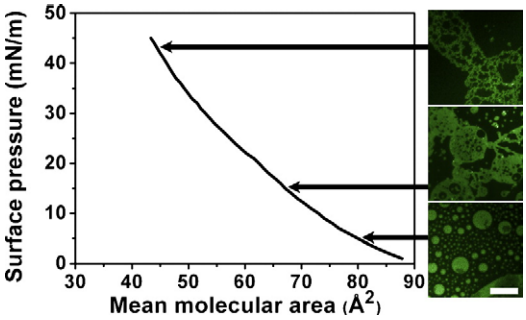

Fig. 1. Compression isotherm of whole myelin at the air/aqueous interface and fluorescence micrographs, representatives of monolayers at low (5 mN/m) medium (12 mN/m) and high (42 mN/m) surface pressure (indicated by arrows). At least two phases are present along the whole compression isotherm. Fractal domains are formed from the circular domains under compression. NBD-PE was used as fluorescent probe. The scale bar is 50 μm in length.

fluorescent lipid probes that partition into liquid-expanded phase domains [24]. Lateral phase separation had also been previously observed in protein-free monolayers mimicking the lipid composition of the erythrocyte membrane [52] and in monolayers made from pulmonary surfactant, a complex lipid–protein mixture secreted from lung alveolar cells [37,38], later confirmed in giant unilamelar vesicles and monolayers made with natural surfactant membranes [53]. A review about the success of the application of monolayer techniques in the latter system has been recently published [54]. Regarding plasma membranes, coexistence of at least two major liquid phases in an interface containing all the components of a natural cell membrane was first described in myelin monolayers [51]. A similar approach using lipids extracted from kidney brush border membranes also showed liquid phase coexistence in monolayers as well as in bilayers [55].

The superstructuring of the myelin monolayer (i.e. shape and size domain distributions), changed upon compression from one morphology dominated by rounded liquid-expanded domains (equilibrium radius $R_e \approx 70$ μm) at low surface pressure to fractal domains formed at higher surface pressures (Fig. 1) in a self-similar pattern lacking a characteristic size (fractal dimension $D_f \approx 1.7$ [56]). The change from the circular pattern to the fractal one does not show a clear transition point and occurs over a range of 10–30 mN/m. The heterogeneity was confirmed later using other probes, or even with no probe at all, by BAM [31]. Sometimes, a third apparently rigid (probably metastable) structure of intermediate fluorescence intensity is observed at very low surface pressure [51].

On the other hand, the protein-free whole lipid extract displayed domains only at very low surface pressure, below 3 mN/m [57]. Continuation of these studies ascertained that our first work reporting domains in monolayers formed with the total lipid extract at relatively high surface pressures [51] was due to some PLP still remaining in the extract. This prompted studies to better understand the participation of the major myelin proteins in establishing the surface domain segregation (see below).

5. What are the coexisting phase domains made of? A partition criterion

The myelin monolayers can be transferred onto silanized glass and immunolabelled against their major proteins (MBP, PLP and CNPase) and some lipids (GM1 ganglioside, cholesterol, galactosylceramide and phosphatidylserine), resulting in a map of the components labelling distribution. This showed two phases in which the labelled components remain within the same domain between 12 and 40 mN/m. Thus, although the distribution and shape of the domains change upon compression, the composition of the phases appears to remain unaltered, at least qualitatively. A single pattern appears to determine

[1] Compact myelin should not be confused with compacted myelin which is a lipid enriched phase with shorter (120–130 130 Å) periodicity induced by decrease of water activity [48].

Table 1
Partition of myelin components into different domains

Component	Phase location	Electrical charge	MW	Physical state "*per se*"
RHO-PE	LE	Negative	1.294	LE
NBD-PE	LE	Negative	0.898	LE
GM1Ganglioside	LE	Negative	1.564	LE
MBP	LE	Positive	18.5/21.5	LE
PLP	LE	Positive	25/29	LE
CNPase	LE	Positive	45/55	Not known
Cholesterol	LO	Neutral	0.387	LC
Galactocerebroside	LO	Neutral	0.728	LC
Phosphatidylserine	LO	Neg. or neutral	0.789	LE-LC

Phase location of the individual components of myelin as determined by immunolabelling after Langmuir–Schaefer film transfer to silanized coverslips. Relevant physicochemical characteristics of the components are shown. MW: molecular weight in KDa. Physical state "*per se*" refers to the physical state of the monolayer of the pure component along the whole compression isotherm (at all surface pressures until collapse) at room temperature.

the component distribution (Table 1). On one hand, there was a phase enriched in labelling of high molecular weight components such as proteins (MBP, PLP, CNPase), GM1 ganglioside and fluorescent lipid probes. Net charges are present in all these components and they are quite compressible (high in-plane elasticity), showing liquid-expanded state *per se* for pure monolayers of MBP [58], PLP[59], GM1 [60] and for the lipid probes used. The other phase was enriched in neutral, lower molecular weight, and rather condensed molecules such as cholesterol [61] and galactosylceramide [60] but also phosphatidylserine, probably condensed by Ca^{2+} present in the subphase buffer [62], and depleted of the major proteins.

It should be noted that heterogeneity in nerve myelin was observed and studied in the past after employing some protocols of tissue fixation for electron microscopy and different physical treatments (temperature, osmotic stress, etc.) [48] In this context, an important coincidence among myelin monolayers and myelin *in vivo* is the presence of two phases, one enriched in lipids, the other one enriched in proteins. Thus, it appears that similar partition criteria acting in myelin monolayers also hold in myelin multilayers, and the loss of membrane asymmetry does not introduce marked changes in the phenomenon of phase segregation. This is probably due to the fact that a major lipid component such as cholesterol, (probably a major factor involved in the establishment of phase segregation in eukaryotic biomembranes in general) is present in both monolayers of natural myelin in high proportion, 0.27 and 0.54 mole fraction for the intra- and extra-cellular monolayer, respectively [48].

6. On the possible physical basis of the partition criteria

Whether the above distribution criteria of condensed and uncharged molecules segregating from expanded and charged molecules should generally hold for other biomembrane monolayers remains an open question. The partition criterion involves various physical parameters such as molecular size, charge, and compressibility. Which of these parameters, and their mutual synergy could be determining the partition of components is also unknown. Actually, the presence of charges in lipids and proteins *per se* should oppose the partition into segregated domains, since the accumulation of equally charged components implies a thermodynamic cost necessary to overcome the electrostatic repulsive energy inside a domain unless divalent counterions are present in the subphase; Ca^{2+} may be an important factor promoting condensation of anionic phospholipids like phosphatidylserine. In the myelin monolayer the major charged components are the basic (positively charged) proteins. Although the charged character may not contribute to the colocalization of proteins, the neutralization of charges could act in this system as a driving force for the partitioning (i.e. as for some lipids with the opposite charge, such as GM1 and the fluorescent lipid probes themselves). Also, the effect of charges may be reduced in the experimental conditions used because the myelin monolayers are formed on a high ionic strength subphase ($I \approx 0.15$ M) compared to water. Actually, in films spread on pure water, in which counter-ion screening is absent and electrostatic effects should be increased, the pattern becomes homogeneous at high surface pressure (Oliveira, R.G. and Maggio, B., unpublished data). Thus, at least for the charged protein components, the presence of charges may not be a direct factor conditioning their selective partitioning but probably an indirect, or secondary, effect correlating with other interactions and properties involved in their segregation.

It might be speculated that the surface compressibility can regulate the partition of components since it is a parameter that includes in it several other factors such as the molecular size, molecular shape, acyl chain-driven partition among domains (saturated and long chains are less compressible compared to unsaturated and short chains), charge effects and configurational restrictions for small molecules. It is well known that small polar molecules, having reduced degrees of freedom tend to be rather condensed. This is the case for cholesterol, fatty acid salts and alcohols [8], ceramide and cerebrosides [28]. Often, the condensed state behaviour results in that the molecules spread at the interface remain self-aggregated in clusters that can be directly observed at the air/water interface, even at 0 mN/m, before starting compression [28]. Additionally, the lack of charge prevents existence of long range lateral electrostatic repulsion at large molecular areas, thus impairing early lift-off of the surface pressure (a manifestation of the repulsive electrostatic force) until a relative closely packed condition is reached upon compression. This is observed in ceramide and cerebroside monolayer isotherms [28,63]. Under these circumstances the lift-off of the isotherm coincides with the merging of the pre-existing condensed domains and crystal periodicities can be measured by GIXD in coexistence with the gas phase [64]. The addition of further sugar residues in the polar head group leads to an increased liquid-expanded character as found for instance in GM1 and other gangliosides [63]. The compressibility of the individual components may represent the balance of several factors that can influence the partition equilibrium of the components. On the other hand, local phenomena such as specific interactions driving close association and/or segregation of components cannot be excluded. H-bonding capabilities among the lipid headgroups would also decrease the compressibility. H-bonding among pure cerebrosides has been reported [65] but subsequent work in binary and ternary lipid mixtures showed that association among glycosphingolipids (present in relatively high proportions in myelin) is, in fact, disfavoured and direct carbohydrate-carbohydrate interactions driving close packing among complex glycosphingolipids is unlikely [66].

The high proportion of cholesterol in the myelin membrane could be at the basis of why the compressibility-based partition criteria may be important. Experiments performed on simple, binary and some ternary systems, indicate that cholesterol enhances the segregation of condensed, from expanded, lipid molecules in different domains. Since cholesterol interacts better with lipids with a relatively high transition temperature, it laterally segregates with them in domains separated from low-melting lipids with unsaturated and/or short hydrocarbon chains [67–70]. In addition, cholesterol is known to interact better with natural sphingolipids [71] that, on average, are more saturated than glycerophospholipids, have a rather long amide-linked chain, and relatively high phase transition temperatures (i.e. Galactosylceramide with a transition temperature above 60 °C). All the molecular properties preferably selected by cholesterol generally correlate with a condensed (low area compressibility) behaviour in monolayers. On the contrary, the unfavourable properties for cholesterol interactions correlate with an expanded (high area compressibility) behaviour. The tendency to associate with those molecules has been related to the organization of closely packed liquid-ordered phases, sometimes described as condensed complexes [72] and superlattices [73]. The resultant liquid-ordered phases have

an increased conformational order (reduced amount of *trans*-gauche isomerizations in favour of trans configuration in the lipid acyl chains, in comparison to liquid-disordered phase), and low lateral elasticity (low area compressibility) [74,75]. For the insertion of molecules such as peptides and proteins, or polymers in general, that penetrate deeply into such phases, the thermodynamic cost required to overcome the cohesive energy needed to create a hole and the elastic energy to deform the membrane will be a major factor [76]. Many transmembrane helices are excluded from cholesterol-rich domains because of the decreased in-plane elasticity [77]. Moreover, the segregation of some proteins from cholesterol-enriched phases has been proposed as a mechanism for domain formation in bilayers [78].

7. The fluid cholesterol-enriched phase

Fluid–fluid immiscibility has been described for cholesterol–phospho(sphingo)lipid mixtures in monolayers [17,72]. In myelin monolayers two fluids are segregated (see Fig. 2); one liquid-expanded phase, with low cholesterol content, and one cholesterol-enriched phase whose main features are: a) the liquid-expanded probe is excluded from this phase; b) nevertheless, this phase is of a very clear fluid nature, as exemplified by: i) the general fluidity of the phase seen under blowing, convection, domain merging and deformation ii) the translational mobility of individual molecules and Brownian motion of included domains, or dust particles [10] iii) the rounded boundaries, a manifestation of the line tension energy minimization, analogous to area minimization in droplets due to surface tension; c) by analogy to bilayer studies, the acyl chains of the associated phospho- and sphingo-lipids are postulated to be ordered in a mostly all-trans configuration, with the chains stretched and perpendicular to the interface [74].

The last point is supported by a marked area condensation induced by cholesterol on the associated lipids in the monolayer phases. Those molecules typically occupy the minimum possible cross sectional area [72,79]. For these reasons, the cholesterol-enriched phase in monolayers is considered analogous to the liquid-ordered phase in bilayers, being the liquid-expanded phase of monolayers equivalent to the liquid-disordered phase of bilayers. The term disordered is due to the gauche-rich conformation of the acyl chains in this phase. Although the configurational state of the acyl chains is not amenable to be explored by BAM, it is very suggestive that the reflectivity of the cholesterol-enriched phase of myelin monolayers remains almost constant over the whole compression isotherm, with a reflectivity change that establishes an upper limit for the relative increase of the optical thickness under compression of 1.3 (by comparison, the LE phase changes its relative optical thickness by a factor of 2.2). That small change of reflectivity means that the thickness of the cholesterol-enriched phase remains almost constant under compression (it is incompressible, just as the individual major components, cholesterol and cerebroside which show rather high constant reflectivity under compression). An implication for this is that the molecular conformation of the components in this phase should not change appreciably under compression, and taking into account that the limiting molecular area of myelin lipids is about 40 $Å^2$ [40], the conformation for the acyl chains in the cholesterol-enriched phase should be quite stretched. This is supported by ellipsometric measurements of the cholesterol-enriched phase, which gives a thickness in the range of 20–30 Å. Although this measurement is not strictly precise the range supports the idea of stretched acyl chains [56]. It is remarkable that perhaps the molecular organization within the liquid-ordered phase is quite insensitive to mechanical disturbances, with the liquid-expanded phase seemingly acting as a buffer to compression–extension stress. Since cholesterol is intercalated between phospho- and glycosphingo-lipids in a liquid-like phase [74] no sharp Bragg peak at 4.2 Å should be expected from X-ray diffraction measurements, coincident with classical studies of natural myelin structure [48]. What is observed in myelin lipids [80] or reconstituted systems with proteins [49,81] as well in nerve myelin [82] is a broad peak centred at 4.6 Å (in fact it goes from 4.4 to 4.8 Å) characteristic of fluid systems including liquid-ordered phase [83,84]. This is consistent with the idea of the existence of liquid-ordered phase both in myelin monolayers or *in vivo*, but is not enough to prove it because this spacing is equal to the one found for the liquid-disordered phase. In this respect, IR spectroscopic determinations could distinguish among these possibilities [85].

8. The many faces of the protein-enriched phase

8.1. The fractal contribution from PLP

The liquid-expanded phase is enriched in proteins and from now on it will be called protein-enriched phase. Under epifluorescence microscopy observation the pattern structure of this phase changes from showing very fluid circular domains (at low surface pressure) to a fractal structure which does not flow easily (at high surface pressure), but still contains liquid-expanded probes. The fractal domains have a characteristic average shape but the size of the cholesterol-enriched lagoons spans from the resolution limit (we cannot discard domains even below this limit) to hundred of micrometers or even millimetres, this being a property of fractal sets [86]. The protein-free purified lipid extract forms a homogeneous phase above 3.0 mN/m. If the lipid mixture contains PLP, the fractal structure of the monolayer is generated (see Fig. 3) even at low surface pressure. The main difference here with whole myelin is that in these films MBP is absent. The partial molecular area of PLP in the mixture remains equal to that of the pure component, which indicates either ideal mixing or no mixing at all [59]. The immunolabelling of the fractal structure demonstrated that the PLP is self-segregated, contributing to the fractal phase [31,57]. Its fractal dimension is similar to the one reported for the fractal present in whole myelin

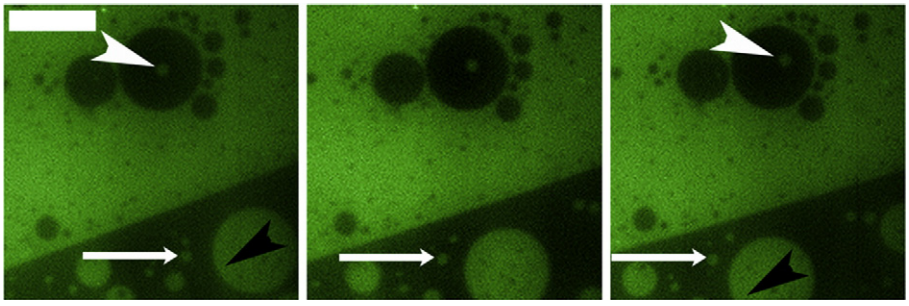

Fig. 2. Fluid–fluid phase coexistence in myelin monolayers (9.6 mN/m). The time elapsed between the pictures is about 10 s. Two kinds of movements can be seen: the directional displacement of the large domain at the bottom (black arrowhead) due to subphase convective flow; the restricted Brownian motion of a small domain (white arrowhead) trapped in a large dark cholesterol-enriched domain at the top. Note the distance to the border is changing over time. Additionally, the displacement of the small domain (white arrow) with respect to the large domain (black arrowhead) can be seen. The scale bar is 50 μm in length.

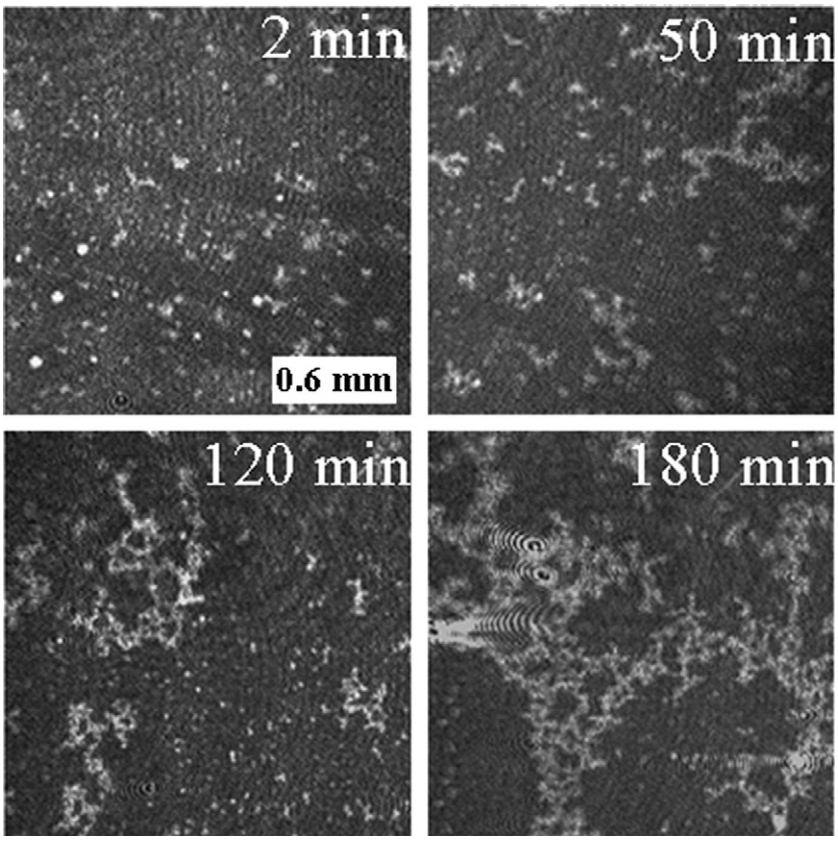

Fig. 3. PLP domain time course evolution, at fixed area and surface pressure (37 mN/m), as seen by BAM in a PLP-whole myelin lipid extract. The brilliant structures are due to PLP segregation. The irreversible growth-aggregation of domains leads to fractal domains.

films and for diffusion limited aggregation (DLA) fractals reported in monolayers [87]. Additionally, different clusters can undergo merging [57] like in diffusion limited cluster aggregation (DLCA) [88]; this process depends on the previous history of the film and is probably the basis behind the non-characteristic length for the domain size. Thus, the organization of the fractal domains depends on the presence of PLP and it is further stabilized when MBP is absent.

8.2. Softening the borders with MBP

In whole myelin monolayers, at low surface pressure, the protein-enriched domains show round boundaries [31], with a theoretically estimated equilibrium radius of 70 μm. Two points should be further explained: a) the domains are polydisperse, as commonly found in monolayer having several components; b) this radius refers to the equilibrium radius at low surface pressure, attained by compression. This (surface pressure dependent) radius results from the balance between two opposing forces: 1) the repulsive dipolar force among molecules in the domain, which makes the domains smaller and causes departure from the circular shape; 2) the line tension that makes the domains larger and circular. At high surface pressure the phase boundaries become increasingly distorted, and domains merge with each other. The high fluidity of the protein-enriched phase, responsible for the rounded domain interfaces depends on the presence of MBP. The protein-free purified lipid extract (as mentioned above) forms a homogeneous mixture above 3 mN/m. After adding MBP (PLP being absent), circular domains are clearly formed; however, fractal morphology is never acquired, and the circular domains remain rather uniformly distributed, because without fractal domain anchors, they are free to relax to a more near-equilibrium distribution. These highly fluorescent MBP-rich domains are in a liquid-expanded state and, according to the partial molecular areas, MBP is ideally interacting with some lipids. It can be deduced that the protein remains associated with a number of lipids that would be equivalent to 2–3 concentric layers surrounding each protein molecule in the film (interestingly, this number is comparable to the amount of lipids perturbed by the protein in the MBP-induced phase separation detected by calorimetry [89] and electron spin resonance [90]). This lipid/protein ratio is maintained even when the proportion of MBP is increased (up to at least sequestering about 40% of the lipid molecules, which is above the proportion of any lipid species segregating with MBP; thus the associated lipid population must be changing its composition, which suggests the lack of selective interactions) [59]. The segregation from the cholesterol-enriched phase may have a major role because the same effect of MBP is observed in simple binary mixtures of palmitoyl sphingomyelin and cholesterol, that is MBP stabilizes the liquid-expanded phase, and segregates from the cholesterol-enriched phase [91]. Also, it may preferentially interact with anionic lipids like GM1 that co-localize with MBP in the Langmuir–Schaefer immunolabellings [31] or other lipids with which MBP has been shown to favourably interact such as sulfatides [43,58,92]. The interesting point is that at 16 mN/m and above, the partial molecular area contribution of MBP to the film area decreases and becomes negligible at 25–30 mN/m, that is, MBP becomes excluded from the monolayer, adopting a more peripheral location [59], as it was early postulated for nerve myelin [48,93], and is the location described in its interaction with glycosphingolipid monolayers and bilayer vesicles [94–96]. A protein reorganization centred at 20 mN/m was inferred from comparing the compression isotherm of whole myelin and myelin lipids [58,40]. This can be most probably ascribed to MBP, which collapses at about 14 mN/m but can be further stabilized by lipid interactions [58]; PLP may also contribute as it undergoes some reorganization centred at about 15 mN/m, without collapsing [59]. At the MBP mole fraction found in myelin, the

rounded borders are disorganized upon compression (like approaching a critical point) and the film becomes homogeneous as revealed by fluorescence microscopy, simultaneously with the exclusion of MBP. It should be noticed that MBP does not desorb from the monolayer since highly reflective protein clusters can be observed by BAM [59]. Also, the liquid-expanded phase domains reappear on expansion under 20 mN/m and MBP remains associated to the Langmuir–Schaefer films of whole myelin even at 42 mN/m [31]. In fact, the MBP-enriched domains can be formed by the spontaneous adsorption of MBP from the subphase to previously compressed myelin lipid films. The Langmuir–Schaefer immunolabelling, the reflectivity measurements and molecular area determination [59] indicate that, when PLP is also present, MBP remains associated with the fractal domains at high surface pressure but in a peripheral location (still associated to but not deeply inserted in the monolayer).

The whole scenario is also consistent with the observation that when phase separation occurs in compact nerve myelin *in vivo*, the compacted phase remains smooth and depleted of intramembranous particles, as well as more ordered, with the typical periodicity of lipid bilayers, being therefore apparently constituted by mostly pure lipids [48]. This compacted phase may be analogous to the cholesterol-enriched phase in monolayers, while PLP, with MBP associated, is located in the other, protein-enriched phase. Additionally, after the compression-driven protein reorganization, the liquid-expanded phase induced by MBP turns into a fractal core of self-segregated PLP [59]; this is compatible with a decreased compressibility of the protein-enriched phase that may no longer act like a buffer for compression.

9. The dynamics of the domain superstructure

The domain superstructure in myelin is surface pressure-sensitive. At low surface pressure, there are two phases: a cholesterol-enriched (liquid-ordered like) phase, which acts as a matrix within which a protein-enriched liquid-expanded phase is distributed. At higher surface pressures (>10–20 mN/m), MBP is increasingly squeezed out from the plane of the monolayer into a sub-surface location; the rounded boundaries become progressively irregular, additionally some protein-enriched domains merge, and finally MBP is peripherally associated to the fractal domains [59]. Myelin proteins appear to act as structuring elements of the lipid fraction. MBP is coupling the pressure changes to both the lateral and transverse surface organization as it is capable of perpendicular displacements into and out of the interface as a result of compression, organizing or disorganizing the liquid phase coexistence [59]. For the highly hydrophobic PLP that remains more fixed in the monolayer, the dynamics of MBP transduces into changes of lateral composition and probably of the viscoelastic properties of the phase surrounding it. PLP forms the core of the fractal domains when MBP leaves the monolayer, and it becomes dispersed into the liquid-expanded phase domains induced by insertion of MBP at lower pressures (See Fig. 4). Similar reorganization processes may be regulated by adsorption of MBP from the subphase [59].

The round-boundary domains associated to MBP are also self-assembled after injection of the protein under a preformed monolayer of myelin lipids. MBP spontaneously acquires its peripheral location under the fractal domains by adsorption [59]. Moreover, whole myelin itself adsorbs at the air/buffer interface developing equivalent surface patterns (according to the surface pressure) to those obtained by spreading and further compression [45]. In the protein–lipid mixtures, as well as in the whole myelin monolayers, the adsorption represents an alternative way to co-spreading and further compression leads to similar domain patterns, suggesting that these represent, at least, near-equilibrium distributions.

For rounded domains, it is obvious that the shape is the one with minimal free energy, and that minimization of the line tension energy determines the border shape [9,10]. The size distribution (being in our case quite polydisperse) most probably represents an out of equilibrium state, but an equilibrium radius of 70 μm for the liquid-expanded domains could be calculated [31] from the theory [10]. The

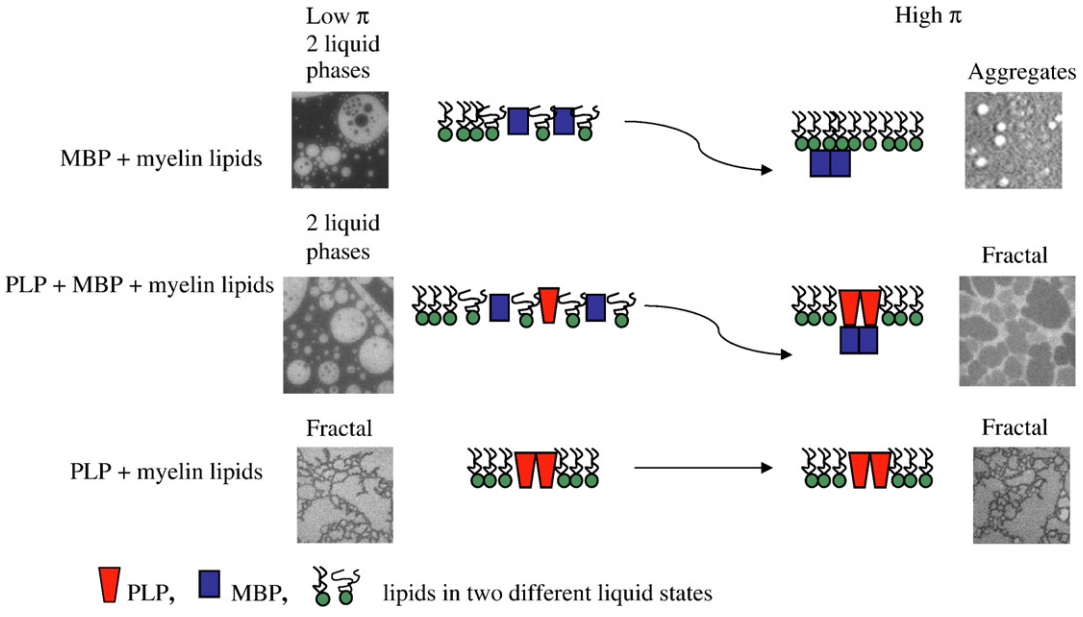

Fig. 4. MBP and PLP contribution to the lateral organization as a function of lateral pressure in myelin monolayers. The individual effects of each protein on the lipid organization are summarized (see text) in the lower and upper panels. The centred panel shows the effect of both proteins when present together in the film. The cartoon represents possible arrangements of the components in each condition. PLP (in the absence of MBP) self-organizes into a fractal pattern, both at low and high surface pressure (lower panel). MBP induces the segregation of two liquid phases at low surface pressure, but it forms aggregates peripherally associated to the lipid monolayer at higher surface pressure (upper panel). When both proteins are present, (at the mole ratio found in myelin) PLP becomes mixed in the MBP-induced liquid phase at low surface pressure (centred panel left). On compression, this liquid phase is disorganized, MBP is slightly squeezed out from the monolayer and PLP becomes surrounded by the lipid phase; in this condition a fractal evolves (centred panel right). All the images are from fluorescence microscopy, except the BAM image showing the MBP aggregates at high surface pressure (upper panel right). Images at low surface pressure were taken at 3.0–5.0 mN/m and the ones at high surface pressure at 30–35 mN/m.

dipole repulsion is effective in preventing merging of the protein-enriched domains at low surface pressure, but under compression the domains begin to merge up to the percolation limit of the protein-enriched domains, which then develop into a fractal structure. In the case of fractal domains the situation is more complicated as it represents a structure remaining in a metastable state over a time scale of at least hours. It should be taken into account that long lived structures out of equilibrium could have relaxation times over a period of weeks [97], the equilibration of the fractal pattern can be neither discarded nor proved. Obviously, the organizing effect of line tension is weakened, but it appears that the dipole repulsion does not dominate the situation since it is weak enough to permit protein-enriched domain fusion [59]. It should be mentioned that the balance between line tension and dipole repulsion is not the only energetic contribution if there is molecular anisotropy or departure from equilibrium (in particular, protein–protein direct interactions cannot be disregarded as a structuring element), which could be the case because the film in the fractal regime becomes very viscous thus impairing shape relaxation. It should be taken into account, that protein–protein interaction could be enhanced due to increased hydrophobic mismatch in comparison to bilayers.

A consequence of the fractal nature of the percolating protein-enriched domains is that the lateral lipid diffusion in the cholesterol-enriched lagoons is corralled, which is one of the factors introducing anomalous diffusion in membranes. Moreover, in these domains not having a characteristic length scale, the lateral propagation of signals is highly variable [56]. A diffusing signal that could propagate over the whole cholesterol-enriched phase at low surface pressure will be restricted to a domain of unpredictable size at higher surface pressure; on the other hand, a signal propagating in a defined circular liquid-expanded domain with a characteristic size will diffuse over the whole system in an irregular percolating cluster at high surface pressure.

10. Monolayer-bilayer correspondence

A biologically relevant question is which, if any, of the superstructures would resemble more closely the one actually present in the membrane *in vivo* (or in bilayer vesicles). One approach to establish this correspondence is based on the equivalence of molecular packing among both arrangements. The molecular packing has been calculated from X-ray diffraction measurement on pure lipid extract without proteins [80]. This displayed a homogeneous phase, as in whole myelin lipid extract monolayers above 3 mN/m. From the stoichiometry of the unit cell, a molecular area of 43.8 $Å^2$ can be calculated. The value points to a laterally compact state. This resembles the close packing that pure lipids would assume at equilibrium in the absence of proteins, similar to the limiting mean molecular area in monolayers of the total lipid fraction from myelin [40].

A second approach is to establish equilibrium among bulk myelin vesicles (or lyophilized or predissolved myelin) and the monolayer or interfacial film at the air/water interface [18]. This renders a surface pressure attained at equilibrium due to the spontaneous adsorption (without any compression work) of the membrane material. This, so-called equilibrium spreading pressure, can be measured and the corresponding molecular area can be taken from the pressure-area isotherm of a compressed film whose molecular area is known. From this measurement (47 mN/m), the more comparable situation to the natural condition is the one at high surface pressure, in fact at the collapsed state, where multilayers begin to form. The fractal pattern can be spontaneously formed and directly observed by adsorption of vesicles from the subphase [45] at the same surface pressures (30–47 mN/m) as found by compression. The advantage of this method is that it allows work, not only with the lipid fraction, but with all the components from the whole membrane. However, it should be noted that there are contradictory results obtained for the equilibrium spreading pressures in different laboratories probably due to differences in the experimental conditions [44]. In fact, the criteria to accomplish at equilibrium is not the equality in molecular area or surface pressure, but the equality of the (electro)chemical potential of each component, so the argument is not strict. The problem of the correspondence among the monolayer and the bilayer states has been a general problem in membrane biophysics. Commonly, the condition of intermolecular packing in monolayers (which is adjusted by the experimentalist) resembling the spontaneous packing adopted in bilayers is thought to be around 30–35 mN/m [98], with considerable fluctuations about this range depending on the surface compressibility [99]. Nevertheless, there is increasing evidence supporting the idea that the reference pressure point relating all equivalent or corresponding surface pressures among the states in the monolayer and the bilayer should be the collapse pressure [100]. At this point, the monolayer does not support further increase on surface pressure and buckles under compression escaping into the third dimension (perpendicular to the monolayer plane). In this sense, since myelin vesicles in the subphase can adsorb to the interface up to the collapse pressure (thus spontaneously forming monolayers in equilibrium with multilayers), myelin appears to support the last scenario [45]. Therefore, the bilayers are in equilibrium with monolayers organized with fractal domain superstructuring and MBP adjacent to the interface. The pattern to be found in bilayers should be a near-equilibrium one, at least for the lipids and MBP adjacent to the interface. Regarding PLP, it is possible that it achieves two different molecular arrangements in equilibrium for the bilayer and monolayer environments but the supramolecular arrangement in bilayers and myelin membrane remain to be explored [49]. A similar close packing of the lipids in nerve myelin and in the monolayers at high surface pressure has been discussed in this section above. The extrinsic (partially immersed) location of MBP in myelin is supported by early evidences [101] and suggests that the monolayer-bilayer thermodynamic equilibrium for MBP corresponds to the arrangement with similar organizations at both interfaces. Near-equilibrium model membrane experiments in monolayers at high surface pressures [58], differential scanning calorimetry in bilayer vesicles [89], and fluorescence measurements [94] all indicated that MBP tends to remain closely associated but with relatively small penetration into the interface, and that it promotes or stabilizes bilayer membrane proximity [94].

Finally, it should be mentioned that in lateral segregation of intramembranous particles observed in early studies of myelin, the proteins frequently arrange in a network-like pattern interspersed with circumscribed particle-free areas of variable dimension [102] as in the fractal structure formed in the myelin monolayers at high surface pressure.

11. The controversy about domains

Myelin heterogeneity (as is also the case of other membranes) has been a matter of controversy. On one hand, myelin under physiological conditions displays small regional specializations, but the bulk of myelin (compact myelin) appears quite homogeneous [48]. This homogeneity is lost under the action of osmotic stress, cooling, divalent cations and several chemicals. It should be noticed that we studied the domain distribution but, as stated above, these domains can be merged into a homogeneous phase and lost in pure water.

On the other hand, detergent extractions propose a scenario in which myelin is heterogeneous, but the composition of the isolated domains depends on the particular technique employed [103–106].

It should be mentioned that many open questions remain about the validity of the protocols used for isolation of detergent insoluble fractions whose discussion is beyond the scope of the present work. On one hand, lowering the temperature from 37 °C to 4 °C represents a considerable environmental disturbance. Particularly, in the old

literature it has been shown that freezing induces structural changes and phase separation in otherwise homogeneous myelin fractions [102,107]. On the other hand, the detergent itself, apart from randomizing the lipid–protein initial organization [108] can induce formation of liquid-ordered phase domains and it has been shown to induce redistribution of gangliosides in whole brain slices [109]. For more detailed discussion and references on the many caveats regarding "raft" domains putatively isolated from natural membranes, and the validity of the basic biophysical features on which their properties are assumed to be based, see [34,108,110-113].

12. Future perspectives

Much convergent evidence has been gathered over the past 30 years indicating that membrane component distribution is sensitive to the environmental factors (temperature, electrolytes, water activity, etc.); even the procedures for domain isolation (cooling for instance) could produce phase separation. How these variables could modulate the phase separation is currently under study. Surely, highly complex multicomponent systems like myelin, will display a multidimensional phase diagram depending on variables like composition (in the membrane and its surroundings) temperature, pH, molecular packing, among others, and these variables can be relatively simple to control in monolayer studies. Particularly, molecular packing, and/or surface pressure, not easy to control in other methodologies, have a profound influence. Two major phases have been described in the past for compact myelin, now we have disclosed the distribution of major myelin components in myelin monolayers. In addition, dynamic structural effects of the individual components were assigned as factors modulating the surface domain microheterogeneity of whole myelin monolayers and of films reconstituted with some of its major lipid and protein fractions.

It is clear that monolayer studies continue to provide important contributions to the understanding of the composition and structuring of membrane segregated domains (and of biomembrane properties in general) under continuously known and controlled surface organization. Also, studies with myelin continue to constitute a highly interesting natural material to unravel at the molecular level the behaviour of biomembranes whose complex dynamics, suggests new and fascinating horizons for research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2008.02.007.

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